The Apparent Transition of a Reovirus 3 Specific Antigen in Murine Lymphoma 2731/L from an Antigenic to a Haptenic Form and the Intracellular Location of the Hapten in the Murine Lymphoma* (32841)

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Stanley *et al.* (1); Keast and Stanley (2); Joske *et al.* (3), and Stanley and Keast (4), have reported on the development of a murine lymphoma 2731/L. This lymphoma developed from spleen cells passaged from a mouse with the chronic disease following neonatal infection with reovirus 3, into neonates (5). Attempts to isolate reovirus 3 from this lymphoma have yielded one virus isolation from an early passage, but subsequent attempts have been unsuccessful (10, 6), although a complement-fixing antigen specific to reovirus 3 and possibly shared with a cultured line of Burkitt's lymphoma cells has been shown to persist through many passages (7).

The evidence presented by Stanley and Keast (4) indicated that the reovirus 3 antigen was fully antigenic in mice growing 2731/L in these early passages, however there was no indiction of its intracellular location in the tumor cell.

We report here on results which show the transition of the reovirus specific antigen from an antigenic to an apparently haptenic form in the mouse and that this presumably "complex hapten" (8) is still fully antigenic in the rabbit and is situated in both the light mitochondrial and microsomal fractions of 2731/L as prepared by sucrose density centrifugation methods.

Materials and Methods. 1. Preparation of the cell fractions of 2731/L. Fractionation at approximately the twentieth transplantation passage of 2731/L lymphoma obtained from over 20 animals which had been inoculated 15 days earlier with approximately 10^6 2731/L cells, was performed using a modification of the differential ultracentrifugation method of De Duve *et al.* (9).

The fractions were sealed in ampuls and stored at —23°C or —70°C. One ampul was used at each inoculation during the immunizing procedure.

Fractions obtained were as follows: (a) Nuclear fraction—electron microscopy showed this to contain nuclei, cell wall, and a small number of intact cells. (b) Mitochondria—electron microscopy indicated that this fraction contained approximately 10% nuclei; 50% larger mitochondria; and some lysosomes and larger polyribosomes. (c) Light mitochondria—electron microscopy indicated that this fraction was made up of approximately 60–80% mitochondria and 40–20% lysosomal particles, polyribosomes, centrioles, and spindle apparatus. (d) Microsomes—electron microscopy indicated that this fraction was made up of 40–50% microsomes, lysosomes, polyribosomes, and a few small mitochondria and centrioles. (e) Postmicrosomes (virus fraction)—electron microscopy indicated that this fraction contained 10–20% microsomes, polyribosomes, and ribosomes. *No virus* particles were seen. (f) Soluble supernate—electron microscopy indicated densely staining material typical of protein under the conditions of staining used.

Groups of 7–10 mice were hyperimmunized by weekly injections, commencing with 0.05 ml and increasing to 0.4 ml after several weeks. Single rabbits were hyperimmunized by the same regime and antigen volumes suitably scaled up.

2. Preparation of 2731/L antigen—for inoculation into rabbits, whole cells were used.

3. Preparation of normal Prince Henry (PH) mouse mesenteric lymph node (NMLN) antigen—for inoculation into rabbits whole cells were used.

4. The preparation of reovirus 3 antigen and antisera in both rabbits and PH mice was identical with that described by Stanley and Keast (7).

5. Adjuvant/antigen preparations; Freund's complete adjuvant was used and a 5:1 mix-

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TABLE I. Complement-Fixing Antibody Titers in Pooled Sera from Several Transplantation Passages of 2731/L Against Test Antigens.

Antigens	Intraperitoneal sublines of 2731/L (ip passage numbers)				
	P3	P5	P9	P13	P15
HEV/PK. 2a	96[a]	24	8	4	2
PK. 2a	2	16	8	4	2
2731/L	96	16	4	128	32
Antibody controls	0	<4	<4	2	<2

[a] Reciprocals of complement-fixation titer.

ture with an antigen was prepared and 0.2 ml volumes inoculated intramuscularly at weekly intervals for several weeks.

6. Mice—PH mice, the "natural" host for 2731/L, were used throughout these experiments.

7. Rabbits—random bred "mongrel" rabbits were used. Prebleed serum was taken and checked against an antigen before the animals were used for any antibody production against the antigen.

8. The complement-fixation test (7)—antigens were prepared by freezing and thawing material 3 times in saline or Hanks' buffered salts solution, followed by centrifugation at 3000 rpm for 30 min. The supernates were designated complement-fixing antigens. The antigens were titered for anticomplementary activity, in the presence of fresh guinea-pig complement, using the checkerboard technique. The antigens were used in the complement fixation test (7) at a dilution where complement titered to the same end point as in the saline control of the checkerboard test. In this series the "neat" preparations fulfilled the above requirements.

9. Hemagglutination-inhibition tests—standard methods were employed. All sera were first heated 56°C for 30 min, kaolin treated, and absorbed with human 'O' cells.

10. Neutralization tests—standard tube neutralization tests were employed. Sera tested here had no pretreatments.

11. Absorption tests—certain sera were absorbed with reovirus 3 prepared by genetron extraction of virus grown for at least 9 passages in a stable porcine cell line, PK.2a. The absorption was with an equal volume of virus preparation overnight at 4°C. The mixture was then spun at 20,000 rpm for 20 min in a Spinco model L refrigerated ultracentrifuge using Rotor 40.

12. Antisera—the following were prepared: (a) Sera against 2731/L antigens: Rabbit anti-2731/L (RA 2731/L); Rabbit antinuclear fraction (RA Nu); Rabbit antilight mitochondrial fraction (RA LM); Rabbit antimicrosomal fraction (RA Mic); Rabbit antipostmicrosomal fraction (RA PM); Rabbit antisoluble supernate (RA S/N); PH mouse antinuclear fraction (PH Nu); PH mouse antilight mitochondrial fraction (PH LM); PH mouse antimicrosomal fraction (PH Mic); PH mouse antipostmicrosomal fraction (PH PM); PH mouse antisoluble supernate fraction (PH S/N); PH mouse antinuclear fraction in Freund's complete adjuvant (PH Nu FA); PH mouse antilight mitochondrial fraction in Freund's complete adjuvant (PH LMFA); PH mouse antimicrosomes in Freund's complete adjuvant (PH MicFA); PH mouse antipostmicrosomal fraction in Freund's complete adjuvant (PH PMFA); and PH mouse antisoluble supernate in Freund's complete adjuvant (PH S/NFA). (b) Sera against other antigens: Rabbit antireovirus 3 ex MEK[1] cells (RA Reo3); Rabbit antinormal PH mouse MLN[2] (RA NMLN); PH mouse anti-Freund's adjuvant (PH FA); and PH mouse antireovirus 3 (PH mouse gut preparation) (PH Reo3).

Results. The results presented in Table I indicate that with serial transplantation of 2731/L, the production of antibody specific for a reovirus 3 antigen decreases to a nonsignificant level, by transplantation passage 15.

[1] MEK = continuous cell line of embryonic kidney cells.

[2] MLN = mesenteric lymph node.

TABLE II. Results of Complement-Fixation Test Titers and Hemagglutination-Inhibition Titers of Sera Prepared in Mice and Rabbits Against Reovirus 3, 2731/L Cells, Cell Fractions of 2731/L Lymphoma Cells, and Normal Mesenteric Lymph Node Cells, and Selected Antigens.[a]

Sera	Serum control	Antigens					HI with Reo3 antigen
		PK. 2a[b]	Reo3/ PK. 2a	2731/L	NPHMLN	MEK	
1. Prebleed RA Reo3	—[b]	—	—	8	12	—	ND
2. RA Reo3 (MEK)	—	8	256	64	12	16	≧160
3. RA 2731/L	4	6	32	>512	1024	4	<10
4. RA 2731/L absorbed with Reo3/PK. 2a	—	—	8	1024	1024	—	ND
5. RA NMLN	8	8	8	256	384	8	ND
6. RA Nu	—	—	—	386	128	—	<10
7. RA LM	4	4	64	>512	384	4	<10
8. RA LM absorbed with Reo3/PK. 2a	8	8	8	768	384	8	ND
9. RA Mic	—	—	16	>512	>512	—	≦10[c]
10. RA Mic absorbed with Reo3/PK. 2a	—	—	—	1024	>512	—	ND
11. RA PM	—	—	—	386	128	—	<10
12. RA S/N	—	—	—	>512	192	—	<10
13. Pooled prebleeds of rabbits	—	—	—	4	—	—	<10
14. Pooled normal PH serum	—	—	—	—	—	—	<10
15. PH Reo3 (PH gut)	—	4	128	8	—	—	<10
16. PH Nu	NT[d]	NT	NT	NT	NT	NT	NT
17. PH LM	—	—	—	—	—	—	<10
18. PH Mic	—	—	—	8	—	—	<10
19. PH PM	—	—	—	—	8	—	<10
20. PH S/N	—	—	—	—	8	—	<10
21. PH NuFA	—	4	16	32	4	ND	<10
22. PH LMFA	—	16	16	32	4	ND	<10
23. PH MicFA	—	4	4	24	24	ND	<10
24. PH PMFA	—	—	—	12	4	ND	<10
25. PH S/NFA	—	—	—	24	—	ND	<10
26. PH FA	—	—	—	32	6	ND	<10

[a] All results are expressed as reciprocals of complement-fixation titers. Controls made up of complement titrations and tests for anti- and procomplementary activity of antigens were included in all runs and were satisfactory before results were recorded.

[b] Abbrev.: — = <4; PK. 2a = porcine stable cells; NPHMLN = normal PH mesenteric lymph node; MEK = monkey embryonic kidney cells; HI = hemagglutination-inhibition; and ND = Not done.

[c] Very slight + reaction here on 2 occasions.

[d] NT = Not tested. This serum was lost during preparation. Because antigens used in its preparation were from the fractionation procedure yielding the other antigens of this series, we could not repeat the immunization procedure. However other test sera of this type have yielded negative results.

Table II summarizes results obtained for the presence of complement-fixing antibody in sera prepared in mice and rabbits against reovirus 3, 2731/L cells and cell fractions from the 2731/L lymphoma against selected antigens. The results of hemagglutination-inhibition tests are also included in this table.

Early work, not recorded, illustrated that the absorption of sera with cell fractions was not feasible. The sera became anticomplementary to high titres. However, anticomplementary activity did not occur when reovirus 3 antigen was used in absorption tests, and so results are based on the absorption from sera of apparently viral specific antibody by viral antigens, rather than the absorption of viral

specific antibody by cell fractions of *the* 2731/L lymphoma.

The results indicate that the complement-fixation antibody specific for reovirus 3, induced in the rabbit by 2731/L cells, is removed by the absorption of the serum with reovirus 3. Similar activity in antisera prepared in the rabbit against the light mitochondrial fraction and the microsomal fraction of the 2731/L lymphoma is also removed by absorption of the sera with reovirus 3 antigen. Fractions of the 2731/L lymphoma other than those of the light mitochondria and microsomal fractions, did not elicit any complement-fixing antibodies directed against reovirus 3 antigen.

Cell fractions of 2731/L inoculated into PH mice did not give rise to any significant production of complement-fixing antibody against reovirus 3, 2731/L or normal MLN antigens.

The mixing of 2731/L cell fractions with Freund's complete adjuvant elicited an antibody response to the 2731/L antigen. Freund's adjuvant alone produced a similar response. This apparently nonspecific response masked any low antibody titer present against specific lymphoma antigens or reovirus 3.

Discussion. We have shown that an "antigen" persists within the 2731/L lymphoma cells which reacts specifically with reovirus 3 antibody (7, 10).

The results presented here show that in the experimental murine system of 2731/L, successive transplantation passages *in vivo* lead to a loss of antibody production directed against a reovirus 3 antigen. This, in conjunction with results recorded in Table II, may be interpreted as a transition of the reovirus 3 antigen from a complete antigenic state to one resembling that of a complex hapten (Tables I and II). However the results of Table I considered alone do not exclude a dilution effect on an adoptive immunization to reovirus 3 perpetrated in the early passages of 2731/L.

In the *in vivo* state the complex hapten remains fully antigenic for the rabbit and cell fractionation studies suggest that the hapten is centred at the light mitochondrial and microsomal fractions (Table II). Electron microscopy of these fractions shows that both contain ribosomal/microsomal material, which may well be the carrier of either reovirus 3 RNA or reovirus 3 specific protein or both. Saline extracts of these fractions will react specifically with reovirus 3 antisera (unpublished observations), and the fractions possess unusual biological activity (4). Furthermore, no complete reovirus 3 particles have been seen in extensive surveys on these fractions or on the postmicrosomal fraction where, under the conditions of fractionation used, complete virus could be located (6).

Fink and Cowles (11) have shown that the choice of host animal is of utmost importance in the search for antigens specific for viruses. Table II illustrates that in the PH mouse, even with the aid of Freund's complete adjuvant, no detectable antibodies were elicited against a reovirus 3 specific antigen from "antigens" prepared from later transplantation passages of 2731/L. The 2731/L lymphoma at the time that these antigens were prepared has been shown to contain an antigen which reacts as a reovirus 3 complement-fixing antigen (7).

Since Wigzell (12), and Uhr and Finkelstein (13) have shown that the concentration of antigen can affect the type of antibody produced, and it is known that threshold values must be exceeded for antibody production to occur, we cannot exclude the possibility of a low level of virus being present, which exceeds the threshold for the rabbit, but not for the mouse. Hemagglutination-inhibition tests using reovirus 3 as the antigen indicate that the antibody produced by the light mitochondrial and microsomal fractions of 2731/L, and directed against reovirus 3, is either heat-sensitive or not of the type normally measured by this test. Two partial reactions at serum dilutions of 1/10 for rabbit antimicrosomal sera (RA Mic) again suggest the production of low level of antibody directed against a reovirus 3 antigen (Table II). Further, no neutralizing activity of these sera against reovirus 3 has so far been demonstrated, although all sera showing any reaction against a reovirus 3 antigen have been extensively tested. Absorption tests (Table II) show that the antibody produced in rabbits and reacting against reovirus 3 is also absorbed from the sera by reovirus 3 in the ap-

propriate circumstances. None of these tests however give any indication of the state of the reovirus 3 antigen. Standard methods for isolation and visualization of virus have proved, with one exception, unsuccessful (10, 6). While these methods may not be sensitive enough to detect low concentrations of reovirus 3, experiments with fluorescent antibody and the enhancement of infectivity by proteolytic enzymes (14) which are in progress may produce information regarding this very important point.

In the light of the postulated viral etiology for Burkitt's lymphoma (15), and the results reported here, we suggest that consideration of the existence of viral information in a complex haptenic form should be considered not only in the case of Burkitt's lymphoma, but in any general concepts of viral etiology for neoplasia. Techniques designed specifically to detect such complex haptens should therefore be routinely applied in the search for viral information contained in neoplastic cells.

Summary. Cell fractionation studies on 2731/L lymphoma cells and the use of these for the production of antisera have shown that a reovirus 3 "antigen" present initially may have undergone a transition into a complex haptenic form during the course of successive transplantation passages. The complex hapten is fully antigenic for the rabbit and using this property, it has been shown to reside in light mitochondrial and microsomal fractions of the 2731/L lymphoma. It is suggested that the hapten is in fact associated with the polyribosomes/microsomes of the lymphoma. The results are discussed with respect to the viral etiology of Burkitt's lymphoma.

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Effect of Exposure Conditions on the Production of Radiation Lesions in Rat Small Intestine (32842)

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Although most investigators (1) have described the morphologic response following irradiation of the small intestine as a uniform generalized phenomenon, there are occasional contradicting reports of uneven morphologic changes even in the same species irradiated